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Comparison of live *Eimeria* vaccination with in-feed salinomycin on growth and immune status in broiler chickens

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ABSTRACT

Coccidiosis vaccines and anticoccidial drugs are commonly used to control *Eimeria* infection during commercial poultry production. The present study was conducted to compare the relative effectiveness of these two disease control strategies in broiler chickens in an experimental research facility. Birds were orally vaccinated with a live, attenuated vaccine (Inovocox), or were provided with in-feed salinomycin (Bio-Cox), and body weights, serum levels of nitric oxide (NO) and antibodies against *Eimeria* profilin and *Clostridium perfringens* PFO proteins, and intestinal levels of cytokine gene transcripts were measured. Vaccinated chickens had increased body weights, greater NO levels, and higher profilin and PFO antibody levels compared with salinomycin-fed birds. Transcripts for interleukin-6 (IL-6), tumor necrosis factor superfamily 15, and interferon-γ were increased, while mRNAs for IL-4 and IL-10 were decreased, in immunized chickens compared with salinomycin-treated chickens. In conclusion, vaccination against avian coccidiosis may be more effective compared with dietary salinomycin for increasing body weight and augmenting pro-inflammatory immune status during commercial poultry production.

1. Introduction

Avian coccidiosis is a globally common parasitic disease that is caused by multiple species of *Eimeria* apicomplexan protozoa infecting the intestinal mucosa. In addition, pre-exposure to some species of *Eimeria* has been strongly implicated in promoting two *Clostridium*-related diseases, necrotic enteritis and gangrenous dermatitis (Williams et al., 2003; Li et al., 2010). *Eimeria* infection of broiler chickens has historically been controlled by vaccination with live parasites or by in-feed coccidiostat drugs (Williams, 2005; Shirley and Lillehoj, 2012). Among the live vaccines approved for use in commercial broilers are virulent (e.g. Inovocox, Coccivac, Immucox, and Advent) and attenuated (e.g. Paracox, Livacox, Viracox, and HatchPak CocciIII) formulations. Anticoccidial drugs can be broadly classified as polyether ionophores which disrupt intracellular osmotic balance (e.g. salinomycin, monensin, lasalocid, and maduramycin) and chemicals which block metabolic pathways (e.g. amprolium, clopidol, decoquinate, and diclazuril) (Allen and Fetterer, 2002).

Conflicting effects on broiler growth performance (body weight gain and feed conversion ratio) have been reported in efficacy trials comparing vaccination- versus drug-based disease control programs. Lehman et al. (2009) found that chickens vaccinated with Coccivac-D had lower weight gains and reduced weight gain-to-feed ratios compared with salinomycin-fed birds within the first 3 weeks post-hatch. Although compensatory growth was seen in the immunized group at later times, overall body weight gains at 8 weeks post-hatch remained higher in the salinomycin-treated chickens. On the contrary, Williams and Gobbi (2002) reported that broilers given a live, attenuated coccidiosis vaccine weighed more than chickens that received an antibiotic growth promoter at 36–37 days (females) and 56 days (males) post-hatch.

Part of the differential effects of vaccination versus pharmacologic medication on growth performance may be related to the different modes of action of these two disease management programs. Chickens infected with *Eimeria* develop protective immunity against re-infection by the homologous parasite (Allen and Fetterer, 2002; Lillehoj et al., 2005). Both cell-mediated immunity, by antigen-specific T lymphocytes and non-specific T cells and macrophages, and humoral immunity by parasite-specific antibodies, play important roles in disease protection, although the rela-

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tive contribution of antibodies remains debated (Lillehoj and Trout, 1996; Song et al., 2000; Ding et al., 2004; Lillehoj et al., 2005). Antibiotic ionophores, on the other hand, are thought to exert a direct cytotoxic effect on coccidia parasites through their ability to facilitate the transport of mono- and divalent cations across the cell membrane to toxic intracellular levels (Callaway et al., 2003). To further define the effects of live parasite vaccination compared with in-feed salinomycin on avian coccidiosis, the current study investigated body weight gain and a variety of immune parameters related to *Eimeria* infection in broiler chickens treated with either management program in an applied poultry research laboratory.

2. Materials and methods

2.1. Experimental design

One-day-old Ross broilers were randomly divided into 12 pens (25 chickens/pen, 6 pens per treatment) with used litter as bedding at the University of Delaware Lasher Laboratory research facility (Georgetown, DE). All broilers were vaccinated *in ovo* against Marek's disease (turkey herpes virus [HVT], SB-1, and Rispen) at 18 days of incubation and post-hatch against Newcastle disease and infectious bronchitis by coarse spray. Chickens in the vaccination group were orally immunized at day 1 with Inovocox as recommended by the manufacturer (Pfizer Animal Health, Madison, NJ) and received a non-medicated basal diet. Chickens in the salinomycin group received the basal diet supplemented with 60 mg/kg of salinomycin (Bio-Cox, Alpharma, Inc., Fort Lee, NJ) for 35 days followed by a non-medicated diet. The basal diet was a mash-type consisting of corn, soybean meal, poultry and animal by-products, and distiller's dried grains soluble. Diet and water were provided *ad libitum*. All experimental protocols were approved by the Small Animal Care Committees of the Beltsville Agricultural Research Center and the University of Delaware.

2.2. Body weight determination and serum samples

At days 14, 28 and 42, chickens were transferred to adjacent room within the research facility for measuring body weight individually and then returned to their respective pens. Immediately after body weight determination, blood was obtained by venipuncture from 5 chickens/pen following euthanasia. Sera were obtained by centrifugation and stored at −20 °C until use.

2.3. Nitric oxide (NO) levels

Serum (100 μl) was mixed with an equal volume of freshly prepared Griess reagent (Sigma, St. Louis, MO) containing 1% (wt/vol) sulfanilamine in 5% phosphoric acid and 0.1% N-naphthylethylenediamine (Lee et al., 2011a). The mixture was incubated for 10 min at room temperature and the optical density at 540 nm was determined with an automated microtiter plate reader (Bio-Rad, Richmond, CA). Nitrite concentrations were calculated from a standard curve generated with $NaNO_2$.

2.4. Antibody levels against Eimeria profilin and Clostridium perfringens PFO

Serum antibody levels were measured by enzyme-linked immunosorbent assay as described (Lee et al., 2011a) using recombinant *Eimeria* profilin protein (Lillehoj et al., 2005) and *C. perfringens* pyruvate:ferredoxin oxidoreductase (PFO) (Lee et al., 2011b). Ninety-six-well microtiter plates were coated overnight with 1.0 μg/well of purified recombinant proteins. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 1% BSA for 1 h at room temperature. Diluted serum samples (1:100, 100 μl/well) were added, incubated with gentle agitation for 2 h at room temperature, and washed with PBS-T. Bound antibodies were detected with horseradish peroxidase-conjugated rabbit anti-chicken IgY secondary antibody (Sigma) and peroxidase substrate. The optical density at 450 nm was determined with an automated microtiter plate reader.

2.5. Intestinal cytokine mRNA levels

A 5-cm segment of the intestine from the mid-jejunum to the ileum was removed at day 28 from 5 chickens/pen. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) as described

Table 1
Oligonucleotide primers used for real time RT-PCR.

Type	RNA	Primer sequence[A]	Amplicon size[B]	Genbank accession no.	References
Reference	GAPDH	F: 5′-GGTGGTGCTAAGCGTGTTAT-3′ R: 5′-ACCTCTGTCATCTCTCCACA-3′	264	K01458	Hong et al. (2006)
Pro-inflammatory	IL-1β	F: 5′-TCTGGGACCACTGTATGCTCT-3′ R: 5′-ACACCAGTGGGAAACAGTATCA-3′	256	AF000631	
	IL-6	F: 5′-CAAGGTGACGGAGGAGGAC-3′ R: 5′-TGGCGAGGAGGGATTTCT-3′	254	AJ309540	
	IL-17F	F: 5′-CTCCGATCCCTTATTCTCCTC-3′ R: 5′-AAGCGGTTGTGGTCCTCAT-3′	292	AJ493595	
	TNFSF15	F: 5′-CCTGAGTATTCCAGCAACGCA-3′ R: 5′-ATCCACCAGCTTGATGTCACTAAC-3′	292	AB194710	Kim et al. (2008)
Th1	IFN-γ	F: 5′-AGCTGACGGTGGACCTATTATT-3′ R: 5′-GGCTTTGCGCTGGATTC-3′	259	Y07922	Hong et al. (2006)
	IL-2	F: 5′-TCTGGGACCACTGTATGCTCT-3′ R: 5′-ACACCAGTGGGAAACAGTATCA-3′	256	AF000631	
	IL-12	F: 5′-AGACTCCAATGGGCAAATGA-3′ R: 5’-CTCTTCGGCAAATGGACAGT-3′	274	NM_213571	
Th2	IL-4	F: 5′-ACCCAGGGCATCCAGAAG-3′ R: 5′-CAGTGCCGGCAAGAAGTT-3′	258	AJ621735	
	IL-10	F: 5′-CGGGAGCTGAGGGTGAA-3′ R: 5′-GTGAAGAAGCGGTGACAGC-3′	272	AJ621614	
	IL-13	F: 5′-CCAGGGCATCCAGAAGC-3′ R: 5′-CAGTGCCGGCAAGAAGTT-3′	256	AJ621735	
Chemokine	IL-8	F: 5′-GGCTTGCTAGGGGAAATGA-3′ R: 5′-AGCTGACTCTGACTAGGAAACTGT-3′	200	AJ009800	

[A] F = forward primer, R = reverse primer.
[B] Size is given in base pairs.

(Hong et al., 2006; Park et al., 2008) and pooled from each treatment group. Five micrograms of total RNA were treated with 1.0 U of DNase I and 1.0 μl of 10× reaction buffer (Sigma), incubated for 15 min at room temperature, 1.0 μl of stop solution was added, and the mixture was heated at 70 °C for 10 min. RNA was reverse transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Quantitative RT-PCR oligonucleotide primers for chicken pro-inflammatory, T helper cell type 1 (Th1), and Th2 cytokines, chemokines, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA from intestine using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene) as described (Hong et al., 2006; Park et al., 2008). Standard curves were generated using $\log_{10}$ diluted standard RNA and the levels of individual transcripts were normalized to those of GAPDH using the Q-gene program (Muller et al., 2002). Each analysis was performed in triplicate. To normalize RNA levels between samples within an experiment, the mean threshold cycle values for the amplification products was calculated by pooling values from all samples in that experiment.

2.6. Statistical analysis

Pen was considered as an experimental unit. Differences between the treatment groups were evaluated by one-way ANOVA. The level of statistical significance was preset at $P < 0.05$.

3. Results

3.1. Body weight measurements

Body weights of Inovocox-vaccinated chickens were greater compared with salinomycin-fed chickens at days 28 and 42 (Table 2).

3.2. NO levels

Serum NO levels in Inovocox-vaccinated chickens were greater compared with salinomycin-fed chickens at days 14, 28, and 42 (Table 3).

Table 2
Effect of coccidiosis vaccination or salinomycin on body weights in grams.

	Vaccine		Salinomycin		*P*-value
	Mean	SD	Mean	SD	
Day 14	359.6[a]	12.0	355.8	13.4	0.619
Day 28	1247.1	14.0	1173.6	51.7	0.020
Day 42	2544.2	123.9	2373.2	131.9	0.043

[a] Values are means and SD ($n = 6$).

Table 3
Effect of coccidiosis vaccination or salinomycin on serum nitric oxide levels.

	Vaccine		Salinomycin		*P*-value
	Mean	SD	Mean	SD	
Day 14	14.1[a]	3.6	9.1	0.8	0.027
Day 28	9.9	1.9	6.9	1.3	0.009
Day 42	13.0	3.3	8.8	2.3	0.041

[a] Values are mean (±SD) micromolecular concentrations of nitric oxide.

Table 4
Effect of coccidiosis vaccination or salinomycin on profilin serum antibody levels.

	Vaccine		Salinomycin		*P*-value
	Mean	SD	Mean	SD	
Day 14	0.45[a]	0.05	0.48	0.04	0.292
Day 28	1.22	0.32	0.76	0.15	0.016
Day 42	1.05	0.20	0.96	0.04	0.349

[a] Values are mean (±SD) of background corrected optical density at 450 nm.

Table 5
Effect of coccidiosis vaccination or salinomycin on PFO serum antibody levels.

	Vaccine		Salinomycin		*P*-value
	Mean	SD	Mean	SD	
Day 14	0.75[a]	0.15	0.58	0.06	0.027
Day 28	0.84	0.08	0.71	0.12	0.041
Day 42	1.12	0.14	0.94	0.07	0.017

[a] Values are mean (±SD) of background corrected optical density at 450 nm.

3.3. Antibody levels against Eimeria profilin and C. perfringens PFO

Profilin serum antibody levels of Inovocox-vaccinated chickens were greater compared with salinomycin-fed chickens at day 28 (Table 4). PFO serum antibody levels of Inovocox-immunized chickens were greater compared with salinomycin-fed chickens at days 14, 28, and 42 (Table 5).

3.4. Intestinal cytokine mRNA levels

The levels of intestinal gene transcripts for IL-1β, IL-2, IL-8, IL-12, IL-13, and IL-17F were identical in the Inovocox-vaccinated chickens compared with salinomycin-fed chickens at day 28 (data not shown). IL-6, TNFSF15, and IFN-γ transcript levels in Inovocox-immunized chickens were greater, while IL-4 and IL-10 transcripts levels were decreased, compared with salinomycin-fed chickens at day 28 (Fig. 1).

4. Discussion

This study was conducted to compare the effects of live parasite vaccination with dietary salinomycin on growth performance and immune status in broiler chickens. In summary, vaccinated chickens had increased body weights, greater serum NO levels, and higher profilin and PFO antibody levels compared with salinomycin-fed birds at 14, 28, and/or 42 days post-hatch. Intestinal gene transcripts for IL-6, TNFSF15, and IFN-γ were increased, whereas IL-4 and IL-10 transcripts were decreased, in immunized chickens compared with salinomycin-fed birds at 28 days. While vaccination and coccidiostat disease management programs have been compared in prior studies with respect to broiler weight gains (Williams, 1999; Williams and Gobbi, 2002; Lee et al., 2009; Lehman et al., 2009), to our knowledge this is the first study highlighting the differential host immune response to these two common coccidiosis control strategies.

The observed increase in body weights at days 28 and 42 in vaccinated versus medicated chickens is consistent with the study by Williams and Gobbi (2002) who reported that broilers immunized with Paracox had greater weight gains after day 36/37 compared with chickens that received the antibiotic growth promoter, avilamycin. No differences in body weights at early time points were seen either in the current study or that by Williams and Gobbi (2002). In contrast, a separate study demonstrated that medicated broilers had greater body weights compared with coccidia-vacci-

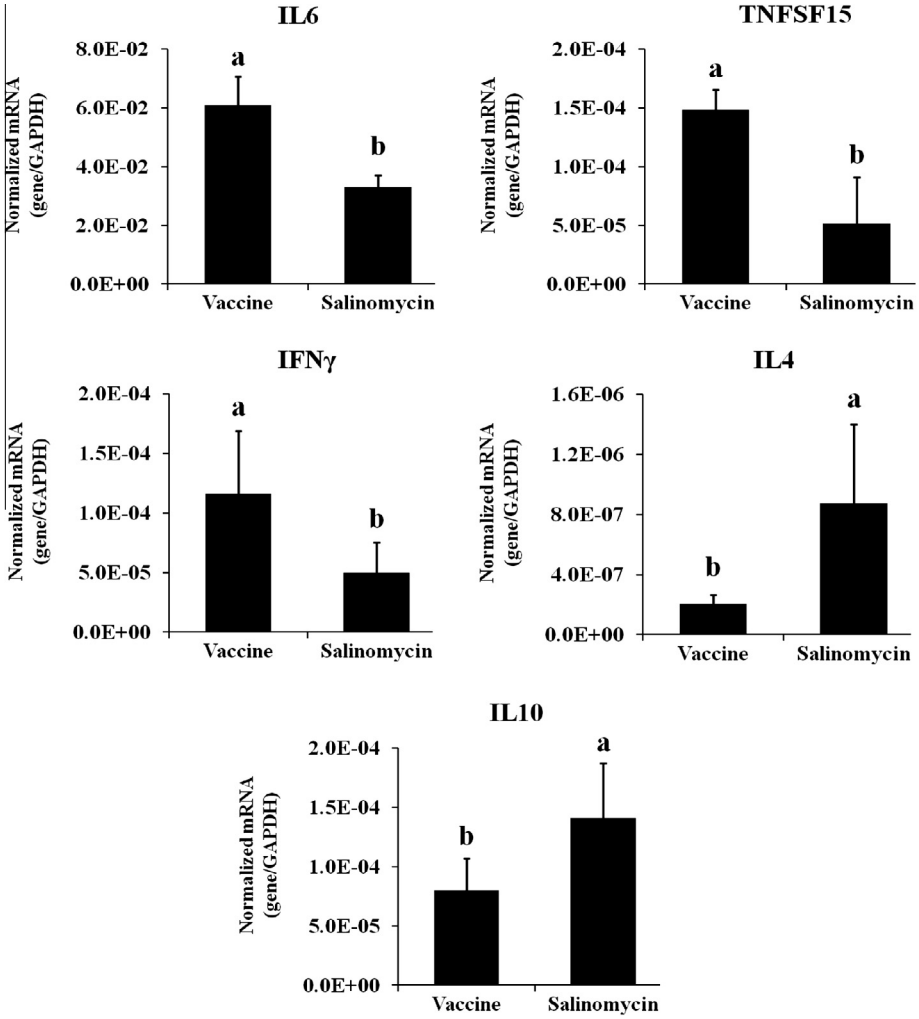

Fig. 1. Effect of coccidiosis vaccination or in-feed salinomycin on intestinal cytokine transcript levels. Chickens were vaccinated with Inovocox or were fed a diet containing salinomycin. At 28 days post-hatch, intestinal tissues were removed and the levels of transcript for IL-6, TNFSF15, IFN-γ, IL-4, IL-10, and GAPDH were quantified by real time RT-PCR. The mRNA levels for each cytokine were normalized to the GAPDH internal control. Vertical bars represent mean ± SD normalized mRNA levels ($n = 6$). Bars with different letters are significantly different ($P < 0.05$).

nated chickens during the first 3 weeks post-hatch (Lehman et al., 2009). It may be possible that antibiotic ionophores promote early weight gain while the effects of vaccination are manifested at later times. It is also apparent that the source of infecting *Eimeria* parasites may influence the extent of weight gain. For example, Jenkins et al. (2010) showed that broilers infected with parasites isolated from poultry farms using live vaccination control and given salinomycin had greater weight gains compared with drug-treated chickens infected with parasites from farms using anticoccidial drugs. This latter study (Jenkins et al., 2010) as well as our finding on lowered body weight gains in salinomycin-fed group may emphasize the presence of drug-resistant *Eimeria* in the litter, leading to reduced drug susceptibility. It is important to note, however, that other reports have been unable to document differences in broiler growth performance between vaccine and anticoccidial drug treatment regimens (Williams et al., 1999; Lee et al., 2009). Thus, future studies should be focused on addressing the relationships between the drug susceptibility and coccidiosis control programs (vaccination vs. medication) on growth performance, which has practical relevance to the poultry industry.

The relative effects of coccidiosis vaccination and in-feed salinomycin on serum levels of NO and specific antibodies, and on intestinal levels of cytokine transcript, may reflect the heightened inflammatory status induced by the live parasites. NO is produced by chicken monocytes and macrophages following exposure to enteric pathogens, such as *Salmonella*, *Clostridium*, and *Eimeria* (Lillehoj and Li, 2004; Babu et al., 2006; Li et al., 2010). Infection with *Eimeria* protozoa also generates an antibody response specifically directed against the profilin protein, and up-regulates the expression of pro-inflammatory cytokines while simultaneously down-regulating the expression of anti-inflammatory cytokines (Engberg et al., 2000; Lillehoj et al., 2004, 2007; Lee et al., 2011c). These combined humoral and cellular immune effects likely reflect host reactions not only to the live coccidia vaccine, but also to infectious *Eimeria* and *Clostridium* microorganisms present in the used litter on which the chickens were raised. Antibiotic ionophores such as salinomycin, on the other hand, are directly cytotoxic for *Eimeria* (Conway et al., 1993) and *C. perfringens* (Engberg et al., 2000) and would, therefore, be expected to decrease parasitic and bacterial intestinal loads and reduce the corresponding host inflammatory responses. Alternatively, salinomycin may activate anti-inflammatory pathways in the avian gut, as evidenced by increased transcription of the counter-regulatory cytokines IL-4 and IL-10, compared with *Eimeria* vaccination. Future studies are needed to test the hypothesis that coccidiosis vaccination skews the immune balance toward a pro-inflammatory/Th1 state, while dietary salinomycin favors an anti-inflammatory status.

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